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Maternal DNA hypomethylation and congenital heart defects

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Abstract

Background—Congenital heart defects (CHDs) are among the most prevalent and serious of birth defects. Multiple maternal factors are thought to contribute to CHD development including folate intake. Maternal DNA methylation, which is dependent on folate metabolism, may impact the risk of CHDs.

Objective—Our study was designed to determine whether maternal long interspersed nucleotide elements-1 (LINE-1) DNA hypomethylation is associated with increased occurrence of non-syndromic CHDs and whether maternal folate-dependent metabolites are correlated with DNA methylation status.

Design—Using a case-control study design, we measured global DNA methylation status among mothers whose pregnancies were affected by non-syndromic CHDs (n=180) and mothers of unaffected pregnancies (n=187). Methylation of LINE-1 was used as a surrogate marker of global DNA methylation status. The association between DNA methylation and CHD risk was determined while adjusting for selected lifestyle factors.

Results—LINE-1 DNA methylation was significantly lower in cases compared with controls (p=0.049). After covariate adjustments, a significant difference between cases and controls remained (p=0.010). Among women with LINE-1 methylation in the lowest decile of DNA methylation, the estimated risk of having a CHD-affected pregnancy was almost twice that of women in all other deciles (OR=1.91; 95% CI: 1.03, 3.58).

Conclusions—Our findings indicate that maternal LINE-1 DNA hypomethylation is associated with an increased risk of CHDs. Future studies investigating the association between maternal DNA methylation patterns and CHDs should be pursued.

Keywords

Congenital heart defects; DNA methylation; LINE-1; folate; maternal biomarkers

INTRODUCTION

Congenital heart defects (CHDs) are the most prevalent and serious of birth defects, occurring in 8 to 10 of every 1,000 live births in the United States (Moller et al., 1993). The molecular mechanisms leading to CHDs are complex, and the majority of cardiac malformations that occur in humans are of unknown etiology. Multiple factors including

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genetic susceptibilities, epigenetic mechanisms, and environmental influences may contribute to the development of non-syndromic CHDs.

Maternal folate deficiency and alterations in one-carbon metabolism have been shown to increase the risk for several birth defects including CHDs (Hobbs et al., 2005; Hobbs et al., 2010; Rosenquist and Finnell, 2001; Shaw et al., 1995; van Driel et al., 2008). During pregnancy there is an increased requirement for folate-dependent nucleotide synthesis and DNA methylation (Oommen et al., 2005), and previous studies suggest that altered DNA methylation may be an underlying mechanism in the development of birth defects (Blom et al., 2006; Li et al., 2005; Okano et al., 1999). Various maternal factors such as diet (Waterland and Jirtle, 2003), genotype (Li et al., 2005), and environmental exposures (Ozanne and Constancia, 2007) have been implicated in abnormal fetal development. These same factors have been shown to affect DNA methylation patterns (Cooney et al., 2002; Candiloro and Dobrovic, 2009; Baccarelli et al., 2009). A combination of maternal genes and environmental exposures may alter the intrauterine environment and thereby modify the fetal phenotype (Furness et al., 2008; MacLennan et al., 2004). In addition to genetic variants, alterations in epigenetic phenomena, such as DNA methylation, could play a crucial role in determining the fetal phenotype. The impact of maternal genetics and epigenetics in the intrauterine environment and subsequent fetal development is not well understood. Little is known about the association between CHDs and maternal DNA methylation despite evidence linking CHDs with maternal folate supplementation and alterations in folate related pathways (Shaw et al., 1995).

DNA methylation is a form of epigenetic gene regulation. The added methyl group can interfere with transcription factor binding, thereby regulating transcription (Comb and Goodman, 1990). Additionally, DNA methylation changes can affect histone modifications and chromatin structure, which can alter gene expression. Hypomethylation of DNA has been reported to contribute to chromosome instability and to alter gene expression, cell differentiation, and apoptosis during embryogenesis (Ehrlich, 2003). Additionally, mutations in the murine DNA methyltransferase (DNMT) gene have been shown to result in embryonic lethality (Li et al., 1992), implicating the importance of DNA methylation in embryogenesis.

The long interspersed nucleotide element (LINE-1) constitutes 17% to 25% of the human genome, with an estimate of over 500,000 LINE-1 elements dispersed throughout the genome (Kazazian, Jr., 2004). Methylation of LINE-1 has been shown to correlate with global DNA methylation (Weisenberger et al., 2005; Yang et al., 2004). Demethylation of these elements, and subsequent activation of retrotransposon activity, can lead to alterations in expression in various genes (Sassaman et al., 1997). LINE-1 methylation of DNA isolated from peripheral blood cells has been shown to be a potential marker of exposure and disease (Bollati et al., 2007; Hsiung et al., 2007).

In the present study we sought to determine if maternal LINE-1 DNA hypomethylation was associated with the occurrence of CHD-affected pregnancies. We also attempted to determine the relationship between LINE-1 methylation and selected metabolites in the homocysteine-methionine pathway.

SUBJECTS AND METHODS

Study population

The National Birth Defects Research and Prevention Study (NBDPS) is an ongoing multi-site population-based case-control study investigating the etiology of 30 non-syndromic birth defects. The NBDPS is the largest case-control study of birth defects ever conducted in

the United States and has been previously described (Yoon et al., 2001). For the current study, case women were Arkansas residents who participated in the NBDPS and who delivered a singleton live birth with a non-syndromic CHD. Cases for whom the pregnancy was also affected by a known single-gene disorder, chromosomal abnormality, or syndrome were excluded from the NBPDS. All diagnostic tests on cardiac NBDPS case infants were reviewed by a pediatric cardiologist to ensure uniform criteria were used for diagnoses. To be eligible for the study described in this manuscript, case infants were required to have at least one cardiac lesion, which included conotruncal, septal, and/or obstructive lesions. Using a classification system developed for the NBDPS, which incorporated three dimensions of cardiac phenotype, cardiac complexity, and extracardiac anomalies (Botto et al., 2007), case women for the current study included those who carried fetuses with simple and association CHDs. Control women were Arkansas residents who had a singleton live birth without birth defects during the same period as the cases, who participated in the NBDPS, and were randomly selected from birth certificate data or hospital discharge logs. Cases and controls spoke either English or Spanish. Blood samples were collected by nurses during home visits in Arkansas that occurred between 2001 and 2008; the research nurses obtained blood samples by venipuncture after acquiring written consent. Blood samples were collected after pregnancy. All participants signed informed consent approved by the University of Arkansas for Medical Sciences Institutional Review Board.

From 548 cases and 243 controls meeting the inclusion criteria, 180 cases and 187 controls were randomly selected using the random number generator in Stata statistical package version 11 (*StataCorp*, College Station, TX).

Covariates

Information regarding selected lifestyle factors was obtained from NBDPS-structured computer-assisted telephone interviews and by in-home interviews conducted at the time of blood draw by a research nurse using a Block Abbreviated Food-Frequency Questionnaire (Block et al., 1990). The information on covariates reflects up to one month prior to the visit. Covariates were selected based on their potential effects on DNA methylation status and the occurrence of heart defects; covariates included were age, race, body mass index (BMI), and the use of multivitamins, cigarettes, and alcohol.

Sample preparation and processing

Fasting blood samples were collected in EDTA-Vacutainer tubes and immediately chilled on ice before they were centrifuged at 4,000 × g for 10 minutes at 4°C to obtain blood plasma. The blood cell pellets and plasma samples were stored at −80°C until DNA isolation and high performance liquid chromatography (HPLC) analyses.

DNA isolation and bisulfite treatment

DNA was isolated from frozen blood samples according to the manufacturer's protocol for the PureGene DNA isolation kit (*Gentra Systems*, Minneapolis, MN). Genomic DNA was quantified using the ABI TaqMan RNase P Quantification Kit (*Applied Biosystems*, Forest City, CA) according to the manufacturer's protocol. Using the EZ DNA Methylation-Direct Kit (*Zymo Research*, Orange Country, CA), 500 ng of genomic DNA underwent sodium bisulfite modification. The converted DNA was resuspended in 20 μl of TE buffer and stored at −80°C until the samples were ready for analysis.

DNA methylation assessment with Methylight

Methylight methodology provides a quantitative methylation-specific PCR assay that allows rapid analysis of methylated and unmethylated alleles via fluorescent detection. The merits

of this technology have been well-characterized (Weisenberger et al., 2005; Ogino et al., 2006; Eads et al., 2000). This process uses bisulfite treatment of DNA, which converts unmethylated cytosines to uracils, leaving the methylated cytosines protected from this conversion (Frommer et al., 1992). Since the primers lack CpG sites, the methylated and unmethylated primer binding sites will be identical after bisulfite conversion. The specificity of the assay occurs during probe hybridization by the use of separately labeled probes. The improved sequence specificity allows relative quantification of methylated and unmethylated alleles in a genomic DNA sample (Zeschnigk et al., 2004). The VIC-labeled probe hybridizes to the sequence of the methylated LINE-1 allele, and the FAM-labeled probe hybridizes to the sequence of the unmethylated LINE-1 allele. Once the probes bind to their selected sites, they are cleaved by Taq polymerase during the PCR reaction, separating the fluorescent tag from the quencher. The amount of resulting fluorescence detected, either VIC or FAM, is directly proportional to the amount of PCR product generated from methylated or unmethylated DNA, respectively (Eads et al., 2000). The probe sequences cover multiple CpG sites within the LINE-1 repetitive sequences, which reflect genome-wide methylation density.

Generation of methylated and unmethylated DNA standards

Methylated and unmethylated standards were generated in order to test the specificity of the PCR reactions. Fully methylated DNA was generated from DNA provided by healthy volunteers. The DNA was incubated with M.SssI enzyme (*New England Biolabs*, Ipswich, MA) at a concentration of 1U/μg of DNA and with 0.16 mM of SAM overnight at 37°C. Extra SAM and M.SssI enzyme was added at the same concentrations for a second overnight incubation at 37°C. The samples were stored at −20°C. To generate unmethylated DNA, healthy volunteer DNA was amplified using the Sigma Whole Genome Amplification (WGA2) kit (*Sigma-Aldrich*, St. Louis, MO). The manufacturer's protocol was used. The product was stored at −20°C. The mixing of methylated and unmethylated DNA as standards for DNA methylation assays has been used successfully by other research groups (Richards, 2006; Yideng et al., 2007).

Real Time Methylation-Specific PCR (Methylight)

PCR reactions were performed with a final reaction volume of 25 μl in 96-well sealed plates on the ABI 7900 HT Real Time PCR System (*Applied Biosystems*, Forest City, CA). Samples contained 12.5 μl of TaqMan Universal Master Mix, 2 μl of bisulfite-treated DNA, 2.5 μM of each of the primers, and 150 nM of TaqMan MGB probes corresponding to the methylated and unmethylated LINE -1 sequence after bisulfite treatment. Real-Time PCR reactions were performed simultaneously on the same 96-well plate. The cycle conditions for the LINE-1 assay were as follows: Initial denaturation at 95°C for 10 minutes to activate Taq polymerase was followed by 50 cycles of denaturation at 94°C for 15 seconds followed by 60°C for 1.5 minutes. Equal PCR efficiency between plates was ensured by including a duplicated sample that contained equal amounts of methylated and unmethylated DNA. The primer and probe sequences are as follows:

Line-1fw, 5′–TTATTAGGGAGTGTTAGATAGTGGG–3′;

Line-1rev, 5′–CCTCTAAACCAAATATAAAAT ATAATCTC–3′;

Line-1met, VIC–TACTTCGACTCGCGCAC GATA-BHQ–3′;

Line-1unmet, 6FAM–CCTACTTCAACTCACACA-BHQ–3′.

Generation of percent DNA methylation values

The amount of methylated and unmethylated DNA was calculated for each sample by interpolation of a standard curve that was established for both methylated and unmethylated

DNA. Serial dilutions of methylated and unmethylated DNA were prepared in order to ensure that all sample values could be interpolated from the standard curve. The percent methylation was calculated by dividing the amount of methylated DNA by the amount of total DNA (methylated plus unmethylated DNA). Each sample was assayed in duplicate and the replicates achieved strong correlation (r=0.84).

Biomarker measurements

Total plasma homocysteine, methionine, SAM, and SAH were determined by HPLC as described elsewhere by our research group (Hobbs et al., 2005). The methodologic details for metabolite elution and electrochemical detection were described previously (Melnyk et al., 1999; Melnyk et al., 2000). Plasma folic acid concentrations were measured by using Quantaphase II radioimmunoassay kit according to the manufacturer's protocol (*Biorad Laboratories*, Hercules, CA).

Statistical analysis

Selected characteristics of case and control subjects were compared with a Fisher's exact test for categorical variables. LINE-1 methylation values for cases and controls were compared using a non-parametric two-sample Wilcoxon rank-sum test. The Wilcoxon rank-sum test was also used to compare LINE-1 methylation between smokers and non-smokers, and between those with normal BMIs (18.5–24.9) and obese individuals (≥30). Multivariable logistic regression was used to compute odds ratios and 95% confidence intervals for the association between LINE-1 methylation and case-control status while adjusting for age, race, vitamin supplementation, smoking, alcohol consumption, and BMI. The frequency distributions of LINE-1 methylation values for cases and controls were graphically examined and compared using a quantile-quantile (Q-Q) plot and a frequency histogram. If the distribution of methylation values was similar for cases and controls, the Q-Q plot would closely follow the diagonal line x=y, whereas deviations from the diagonal line indicate different distributions of methylation values between cases and controls. The relationships between LINE-1 DNA methylation and selected homocysteine-methionine pathway biomarkers were determined by computing Spearman's partial correlation coefficient after covariate adjustments. The comparison between cases and controls for selected biomarkers was conducted using a multiple linear regression model adjusting for the covariates previously listed.

Analyses were performed using the Stata statistical package version 11 (*StataCorp*, College Station, TX).

RESULTS

Study population

LINE-1 DNA methylation was measured in 367 participants (180 cases and 187 controls). Table 1 presents selected characteristics of study participants. The majority of cases (61.7%) and controls (60.4%) were less than 30 years old. The study population consisted mostly of Caucasian women. The frequencies of most characteristics were similar between cases and controls. However, smoking was significantly more prevalent in cases (30.0%) than in controls (18.7%; p=0.015), and the median time interval between the end of pregnancy and blood draw was significantly longer for controls (16.5 months; range = 0.13–65.3 months) than for cases (15.4 months; range = 4.3–63.3 months; p=0.035). Additional analyses to determine if smoking and the interval between end of pregnancy and blood draw were confounders was conducted. Specifically, neither smoking nor time of blood draw was associated with LINE-1 methylation status in either cases, controls, or both (data not shown).

Selected metabolite analysis

Plasma concentrations of selected metabolites involved in one-carbon metabolism are summarized in Table 2. As we have previously reported, we observed significant differences between cases and controls for the biomarkers analyzed after adjusting for multiple covariates. When compared to controls, cases had higher mean concentrations of homocysteine and SAH and lower mean concentrations of methionine, SAM, the SAM/SAH ratio, and folate. The metabolite concentrations in the cases were all reflective of a decreased cellular methylation capacity.

LINE-1 DNA methylation analysis

Table 3 presents summary statistics of LINE-1 DNA methylation in cases and controls. DNA methylation was significantly lower (hypomethylation) in cases when compared to controls (p=0.049). To control for potential confounders, we constructed a model to adjust for age, race, body mass index (BMI), and the use of multivitamins, cigarettes, and alcohol. The significant association between LINE-1 methylation and case/control status remained after covariate adjustment (p=0.010).

To further explore the relationship between LINE-1 methylation and CHDs in our population, we conducted analyses stratified for smoking and obesity. Smoking was more prevalent among cases than controls, and thus we stratified our analyses by smoking status. We did not observe a significant relationship among LINE-1 methylation and smoking status (p=0.974). We also compared LINE-1 methylation among individuals with a normal BMI and obese individuals, but the result was not statistically significant (p=0.557).

To determine if the associations between DNA methylation and cardiac defects were related to specific cardiac phenotypes, an analysis stratified by cardiac phenotypes was conducted. Cardiac phenotypes included conotruncal, septal, and obstructive defects. Cases that had more than one cardiac defect type were included in all relevant strata. Due to a small number of cases, the 14 cases that did not fall within one of the main defect groups were excluded from the stratified analysis. Among cases, 47.2% had atrial or ventricular septal defects, 35.4% had right- and left-sided obstructive defects, and 16.7% had conotruncal defects.

After covariate adjustment, LINE-1 methylation was significantly lower among women with pregnancies affected by septal defects (mean=81.26, SD=11.44) compared to controls (mean=83.64, SD=10.16; p=0.043). LINE-1 methylation was lower among conotruncal and obstructive cases compared to controls (p=0.085 and 0.086, respectively), but these results did not achieve statistical significance. LINE-1 methylation was lowest among conotruncal cases (mean=80.0%, SD=11.2). Because LINE-1 methylation did not deviate significantly between cardiac phenotypes, cases were combined for subsequent analyses to maximize power to test study hypotheses. A Q-Q plot and histograms were created to examine and compare the frequency distributions of LINE-1 methylation in cases and controls (Figure 1). Both the Q-Q plot and the histogram revealed marked deviations in methylation between cases and controls at the lower values of the distribution.

Based on these plots, LINE-1 DNA methylation was further compared between cases and controls by computing adjusted odds ratios and 95% confidence intervals at various cutoffs on the basis of methylation levels within controls (Table 4).

Of the 180 cases, 55 (30.6%) cases had with LINE-1 methylation values below the 20th percentile of controls and 33 (18.3%) cases had LINE-1 methylation values below the 10th percentile of controls, indicating a shift to lower values in LINE-1 methylation in cases relative to controls. Women in this bottom decile were almost twice as likely to have a

CHD-affected pregnancy compared to women above this decile (OR= 1.91; 95% CI: 1.03, 3.58).

These findings suggested that a lower DNA methylation status may be associated with an increased risk of CHDs.

LINE-1 methylation and selected metabolites correlation

The correlations between LINE-1 DNA methylation and selected plasma metabolites were computed to determine if metabolites were predictive of DNA methylation status among cases and controls (Table 5). Homocysteine had the strongest correlation with LINE-1 methylation. As LINE-1 DNA methylation decreased, total homocysteine concentration increased, resulting in a negative correlation (Spearman's $r=-0.135$, $p=0.013$). A separate model for cases and controls correlating LINE-1 and homocysteine was computed for cases and controls. The slopes from each model were not significantly different (data not shown). Concentrations of methionine, s-adenosylmethionine (SAM), s-adenosylhomocysteine (SAH), and folate were not significantly correlated with LINE-1 methylation status.

DISCUSSION

To our knowledge, our findings represent the first published study in which DNA methylation of LINE-1 was compared between mothers of CHD-affected pregnancies and controls. DNA hypomethylation of the LINE-1 repetitive element was observed in case mothers when compared to control mothers. Placement in the lowest deciles of methylation resulted in an increased risk of CHD-affected pregnancies. LINE-1 DNA methylation status was not strongly correlated with selected methionine pathway plasma metabolites.

The magnitude of difference between case and control LINE-1 methylation was relatively small compared to differences reported in other disease models, for example, between normal and tumor tissue samples (Iacopetta et al., 2007). However, a small magnitude of difference in peripheral blood LINE-1 methylation was observed in a previous case/control study (Hsiung et al., 2007). During pregnancy, increases in cellular proliferation and one-carbon metabolism are required for uterine enlargement, expansion of blood volume, placental development, and fetal growth (Bruinse and van den, 1995; Tamura and Picciano, 2006). For the developing embryo, the increased demand for methyl groups for nucleotide synthesis and DNA methylation may result in a small threshold between sufficient and insufficient methylation capacity. Insufficient methylation capacity may cause deleterious changes in developmental programming resulting in CHDs and other birth defects.

LINE-1 methylation has shown to be significantly correlated with global methylation levels measured by HPLC in cancer models. Additionally, demethylation of repetitive elements can ultimately lead to aberrant expression in other genes (Asada et al., 2006). It has been suggested that hypomethylation of repetitive elements may influence genomic instability and may consequently cause genome instability in the progeny (Filkowski et al., 2010). Although speculation regarding transgenerational epigenetic inheritance exists, the majority of evidence exists in animal models (Morgan and Whitelaw, 2008). Correlations between parent-child pairs in LINE-1 methylation suggest the possibility of transgenerational epigenetic inheritance being associated with disease risk (Mirabello et al., 2010).

A decrease in the incidence of CHDs and neural tube defects (NTDs) can be achieved with preconception folic acid supplementation (Hobbs et al., 2010). The shared biological sensitivity for these separate anatomical sites is thought to involve their common neural epithelial cell origin (Rosenquist and Finnell, 2001). Thus, CHDs and NTDs may share multiple similar pathophysiological pathways. It has been postulated that inhibition of the

methylation cycle may contribute to the development of NTDs through the disruption of crucial reactions involving the methylation of DNA and other biomolecules (Dunlevy et al., 2006). Recent studies have shown an increased risk of NTDs with decreased LINE-1 and global methylation status (Chen et al., 2010; Wang et al., 2010). These studies provide evidence that genomic methylation may play a role in the development of NTDs. Additional studies to confirm the role of DNA methylation in CHD risk should be pursued as well.

The LINE-1 repetitive element has been used as a measure for genomic methylation status for methylation-specific PCR assays (Hsiung et al., 2007; Iacopetta et al., 2007; Yideng et al., 2007) and widely used in pyrosequencing assays (Wang et al., 2010; Figueiredo et al., 2009; Bollati et al., 2007). LINE-1 methylation patterns appear to be tissue-specific and vary depending on cell type (Chalitchagorn et al., 2004), thus the values obtained from methylation studies may vary greatly depending on the processed biological sample and the assay performed. The range of LINE-1 methylation values presented in this study are consistent with two previously published studies assessing LINE-1 methylation (Iacopetta et al., 2007; Wilhelm et al., 2010). We observed a large proportion of LINE-1 methylation values greater than 80%, and this observation is in agreement with other published studies (Iacopetta et al., 2007; Jin et al., 2009; Richards et al., 2009).

An inverse correlation between cord plasma homocysteine and LINE-1 methylation in humans has been recently shown (Fryer et al., 2009). We also observed a modest inverse correlation between LINE-1 methylation and plasma homocysteine. The lack of a strong correlation between LINE-1 methylation and selected biomarkers may be explained by the hypothesis proposed by Kim (Kim, 2005) stating that the effects of folate deficiency and the supply of DNA methyl groups may be gene- and site-specific and dependent on the cell type, organ, stage of transformation, and degree and duration of folate depletion. Previous studies have also shown a lack of correlation between LINE-1 methylation and methionine pathway metabolites (Figueiredo et al., 2009; Wang et al., 2010). Since folate pathway metabolites lacked strong correlation with LINE-1 methylation, we postulate that the genomic methylation differences observed may be due to other unmeasured factors. Single nucleotide polymorphisms, additional metabolites, and environmental exposures are factors that may explain the alterations in DNA methylation observed in the study.

Certain methodological limitations of this study should be considered. Subjects were enrolled in a case-control study after completion of pregnancy. Given the rare prevalence of CHDs, the resources and sample size required to conduct a cohort study among women enrolled before conception and followed to the completion of pregnancy are enormous; thus, case-control design is the preferred epidemiological design for rare outcomes. Thus, we did not measure DNA methylation changes during pregnancy and fetal organogenesis, when changes in methylation status would be expected to have the greatest effect. We have previously stated that the plasma biomarker concentrations in our study design may not represent biomarker concentrations at the time of organogenesis, but may identify women who have persistent metabolic imbalances (Hobbs et al., 2005). It has been shown that homocysteine concentrations and dietary patterns remain relatively stable from preconception throughout pregnancy and postpartum (Cikot et al., 2001; Cuco et al., 2006). We found no correlation between maternal age and LINE-1 methylation (data not shown). Thus, LINE-1 methylation observed after pregnancy may reflect stable adult profiles that were present at the time of conception. Another limitation of our experimental design is that only allowed the analysis of only fully methylated and unmethylated alleles in the LINE-1 repetitive element. If partial methylation occurs at the level of probe discrimination, the sequence derived after bisulfite conversion is recognized by neither of the two probes. Thus, partial methylation may affect the ability of the assay to detect global methylation density via the amplification of the LINE-1 consensus sequence. This design is in contrast to other

methods that measure the methylation level at several single CpG sites. An advantage of Methylight is its high sensitivity and low input DNA requirement, but it is prone to higher variability that other methods (Aparicio et al., 2009).

To our knowledge, our study is the first to measure maternal DNA methylation in a case-control study of CHDs. The study has notable strengths including the analysis of DNA methylation in a relatively large population of mothers in which information regarding various lifestyle factors and plasma metabolite concentrations were available. Our study design allowed the assessment of the relationship between LINE-1 DNA methylation and the risk of CHDs. Our findings indicate maternal LINE-1 hypomethylation is associated with an increased risk of CHD-affected pregnancies. Alterations in maternal DNA methylation converge with evidence from previous studies that indicate folate-dependent genetic and metabolic susceptibilities increase the risk of CHDs. These results suggest that future studies involving repetitive, global, and gene-specific maternal DNA methylation and CHDs are warranted. The ability to possibly restore aberrant DNA methylation to normal levels via nutritional intervention makes DNA methylation an attractive potential therapeutic target. Identification of genetic and epigenetic profiles associated with an increased risk of CHDs may add new dimensions to assessing preconception risk and may prove to be instrumental in elucidating the mechanisms that underlie the development of CHDs.

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Reference List

Aparicio A, North B, Barske L, Wang X, Bollati V, Weisenberger D, Yoo C, Tannir N, Horne E, Groshen S, Jones P, Yang A, Issa JP. LINE-1 methylation in plasma DNA as a biomarker of activity of DNA methylation inhibitors in patients with solid tumors. Epigenetics. 2009; 4:176–184. [PubMed: 19421002]

Asada K, Kotake Y, Asada R, Saunders D, Broyles RH, Towner RA, Fukui H, Floyd RA. LINE-1 hypomethylation in a choline-deficiency-induced liver cancer in rats: dependence on feeding period. J Biomed.Biotechnol. 2006; 2006:17142. [PubMed: 16877811]

Baccarelli A, Wright RO, Bollati V, Tarantini L, Litonjua AA, Suh HH, Zanobetti A, Sparrow D, Vokonas PS, Schwartz J. Rapid DNA Methylation Changes after Exposure to Traffic Particles. Am.J Respir.Crit Care Med. 2009

Block G, Hartman AM, Naughton D. A reduced dietary questionnaire: development and validation. Epidemiology. 1990; 1:58–64. [PubMed: 2081241]

Blom HJ, Shaw GM, den HM, Finnell RH. Neural tube defects and folate: case far from closed. Nat.Rev.Neurosci. 2006; 7:724–731. [PubMed: 16924261]

Bollati V, Baccarelli A, Hou L, Bonzini M, Fustinoni S, Cavallo D, Byun HM, Jiang J, Marinelli B, Pesatori AC, Bertazzi PA, Yang AS. Changes in DNA methylation patterns in subjects exposed to low-dose benzene. Cancer Res. 2007; 67:876–880. [PubMed: 17283117]

Botto LD, Lin AE, Riehle-Colarusso T, Malik S, Correa A. Seeking causes:Classifying and evaluating congenital heart defects in etiologic studies. Birth Defects Res.A Clin Mol.Teratol. 2007; 79:714–727. [PubMed: 17729292]

Bruinse HW, van den BH. Changes of some vitamin levels during and after normal pregnancy. Eur J Obstet.Gynecol.Reprod.Biol. 1995; 61:31–37. [PubMed: 8549845]

Candiloro IL, Dobrovic A. Detection of MGMT promoter methylation in normal individuals is strongly associated with the T allele of the rs16906252 MGMT promoter single nucleotide polymorphism. Cancer Prev.Res (Phila Pa). 2009; 2:862–867.

Chalitchagorn K, Shuangshoti S, Hourpai N, Kongruttanachok N, Tangkijvanich P, Thong-ngam D, Voravud N, Sriuranpong V, Mutirangura A. Distinctive pattern of LINE-1 methylation level in normal tissues and the association with carcinogenesis. Oncogene. 2004; 23:8841–8846. [PubMed: 15480421]

Chen X, Guo J, Lei Y, Zou J, Lu X, Bao Y, Wu L, Wu J, Zheng X, Shen Y, Wu BL, Zhang T. Global DNA hypomethylation is associated with NTD-affected pregnancy: A case-control study. Birth Defects Res A Clin Mol.Teratol. 2010; 88:575–581. [PubMed: 20641100]

Cikot RJ, Steegers-Theunissen RP, Thomas CM, de Boo TM, Merkus HM, Steegers EA. Longitudinal vitamin and homocysteine levels in normal pregnancy. Br.J Nutr. 2001; 85:49–58. [PubMed: 11227033]

Comb M, Goodman HM. CpG methylation inhibits proenkephalin gene expression and binding of the transcription factor AP-2. Nucleic Acids Res. 1990; 18:3975–3982. [PubMed: 1695733]

Cooney CA, Dave AA, Wolff GL. Maternal methyl supplements in mice affect epigenetic variation and DNA methylation of offspring. J Nutr. 2002; 132:2393S–2400S. [PubMed: 12163699]

Cuco G, Fernandez-Ballart J, Sala J, Viladrich C, Iranzo R, Vila J, Arija V. Dietary patterns and associated lifestyles in preconception, pregnancy and postpartum. Eur J Clin Nutr. 2006; 60:364–371. [PubMed: 16340954]

Dunlevy LP, Burren KA, Mills K, Chitty LS, Copp AJ, Greene ND. Integrity of the methylation cycle is essential for mammalian neural tube closure. Birth Defects Res A Clin Mol.Teratol. 2006; 76:544–552. [PubMed: 16933307]

Eads CA, Danenberg KD, Kawakami K, Saltz LB, Blake C, Shibata D, Danenberg PV, Laird PW. MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res. 2000; 28:E32. [PubMed: 10734209]

Ehrlich M. Expression of various genes is controlled by DNA methylation during mammalian development. J Cell Biochem. 2003; 88:899–910. [PubMed: 12616529]

Figueiredo JC, Grau MV, Wallace K, Levine AJ, Shen L, Hamdan R, Chen X, Bresalier RS, Keown-Eyssen G, Haile RW, Baron JA, Issa JP. Global DNA hypomethylation (LINE-1) in the normal colon and lifestyle characteristics and dietary and genetic factors. Cancer Epidemiol.Biomarkers Prev. 2009; 18:1041–1049. [PubMed: 19336559]

Filkowski JN, Ilnytskyy Y, Tamminga J, Koturbash I, Golubov A, Bagnyukova T, Pogribny IP, Kovalchuk O. Hypomethylation and genome instability in the germline of exposed parents and their progeny is associated with altered miRNA expression. Carcinogenesis. 2010; 31:1110–1115. [PubMed: 19959559]

Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc.Natl.Acad.Sci.U.S.A. 1992; 89:1827–1831. [PubMed: 1542678]

Fryer AA, Nafee TM, Ismail KM, Carroll WD, Emes RD, Farrell WE. LINE-1 DNA methylation is inversely correlated with cord plasma homocysteine in man: a preliminary study. Epigenetics. 2009; 4:394–398. [PubMed: 19755846]

Furness DL, Fenech MF, Khong YT, Romero R, Dekker GA. One-carbon metabolism enzyme polymorphisms and uteroplacental insufficiency. Am J Obstet Gynecol. 2008; 199:276–278. [PubMed: 18771981]

Hobbs CA, Cleves MA, Melnyk S, Zhao W, James SJ. Congenital heart defects and abnormal maternal biomarkers of methionine and homocysteine metabolism. Am.J.Clin.Nutr. 2005; 81:147–153. [PubMed: 15640474]

Hobbs, CA.; Shaw, GM.; Werler, MM.; Mosley, BS. Folate Status and Birth Defect Risk: Epidemiological Perspective. In: Bailey, LB., editor. Folate in Health and Disease. Second Edition. Boca Raton FL: CRC Press; 2010. p. 133-154.

Hsiung DT, Marsit CJ, Houseman EA, Eddy K, Furniss CS, McClean MD, Kelsey KT. Global DNA methylation level in whole blood as a biomarker in head and neck squamous cell carcinoma. Cancer Epidemiol.Biomarkers Prev. 2007; 16:108–114. [PubMed: 17220338]

Iacopetta B, Grieu F, Phillips M, Ruszkiewicz A, Moore J, Minamoto T, Kawakami K. Methylation levels of LINE-1 repeats and CpG island loci are inversely related in normal colonic mucosa. Cancer Sci. 2007; 98:1454–1460. [PubMed: 17640302]

Jin M, Kawakami K, Fukui Y, Tsukioka S, Oda M, Watanabe G, Takechi T, Oka T, Minamoto T. Different histological types of non-small cell lung cancer have distinct folate and DNA methylation levels. Cancer Sci. 2009; 100:2325–2330. [PubMed: 19764999]

Kazazian HH Jr. Mobile elements: drivers of genome evolution. Science. 2004; 303:1626–1632. [PubMed: 15016989]

Kim YI. Nutritional epigenetics: impact of folate deficiency on DNA methylation and colon cancer susceptibility. J Nutr. 2005; 135:2703–2709. [PubMed: 16251634]

Li D, Pickell L, Liu Y, Wu Q, Cohn JS, Rozen R. Maternal methylenetetrahydrofolate reductase deficiency and low dietary folate lead to adverse reproductive outcomes and congenital heart defects in mice. Am J Clin Nutr. 2005; 82:188–195. [PubMed: 16002818]

Li E, Bestor TH, Jaenisch R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell. 1992; 69:915–926. [PubMed: 1606615]

MacLennan NK, James SJ, Melnyk S, Piroozi A, Jernigan S, Hsu JL, Janke SM, Pham TD, Lane RH. Uteroplacental insufficiency alters DNA methylation, one-carbon metabolism, and histone acetylation in IUGR rats. Physiol Genomics. 2004; 18:43–50. [PubMed: 15084713]

Melnyk S, Pogribna M, Pogribny I, Hine RJ, James SJ. A new HPLC method for the simultaneous determination of oxidized and reduced plasma aminothiols using coulometric electrochemical detection. J.Nutr.Biochem. 1999; 10:490–497. [PubMed: 15539328]

Melnyk S, Pogribna M, Pogribny IP, Yi P, James SJ. Measurement of plasma and intracellular S-adenosylmethionine and S-adenosylhomocysteine utilizing coulometric electrochemical detection: alterations with plasma homocysteine and pyridoxal 5'-phosphate concentrations. Clin.Chem. 2000; 46:265–272. [PubMed: 10657384]

Mirabello L, Savage SA, Korde L, Gadalla SM, Greene MH. LINE-1 methylation is inherited in familial testicular cancer kindreds. BMC.Med Genet. 2010; 11:77. [PubMed: 20478068]

Moller JH, Allen HD, Clark EB, Dajani AS, Golden A, Hayman LL, Lauer RM, Marmer EL, McAnulty JH, Opari S. Report of the task force on children and youth. American Heart Association. Circulation. 1993; 88:2479–2486.

Morgan DK, Whitelaw E. The case for transgenerational epigenetic inheritance in humans. Mamm.Genome. 2008; 19:394–397. [PubMed: 18663528]

Ogino S, Kawasaki T, Brahmandam M, Cantor M, Kirkner GJ, Spiegelman D, Makrigiorgos GM, Weisenberger DJ, Laird PW, Loda M, Fuchs CS. Precision and performance characteristics of bisulfite conversion and real-time PCR (MethyLight) for quantitative DNA methylation analysis. J Mol.Diagn. 2006; 8:209–217. [PubMed: 16645207]

Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell. 1999; 99:247–257. [PubMed: 10555141]

Oommen AM, Griffin JB, Sarath G, Zempleni J. Roles for nutrients in epigenetic events. J Nutr.Biochem. 2005; 16:74–77. [PubMed: 15681164]

Ozanne SE, Constancia M. Mechanisms of disease: the developmental origins of disease and the role of the epigenotype. Nat.Clin Pract.Endocrinol.Metab. 2007; 3:539–546. [PubMed: 17581623]

Richards EJ. Inherited epigenetic variation--revisiting soft inheritance. Nat.Rev.Genet. 2006; 7:395–401. [PubMed: 16534512]

Richards KL, Zhang B, Baggerly KA, Colella S, Lang JC, Schuller DE, Krahe R. Genome-wide hypomethylation in head and neck cancer is more pronounced in HPV-negative tumors and is associated with genomic instability. PLoS.ONE. 2009; 4:e4941. [PubMed: 19293934]

Rosenquist TH, Finnell RH. Genes, folate and homocysteine in embryonic development. Proc.Nutr.Soc. 2001; 60:53–61. [PubMed: 11310424]

Sassaman DM, Dombroski BA, Moran JV, Kimberland ML, Naas TP, DeBerardinis RJ, Gabriel A, Swergold GD, Kazazian HH Jr. Many human L1 elements are capable of retrotransposition. Nat.Genet. 1997; 16:37–43. [PubMed: 9140393]

Shaw GM, O'Malley CD, Wasserman CR, Tolarova MM, Lammer EJ. Maternal periconceptional use of multivitamins and reduced risk for conotruncal heart defects and limb deficiencies among offspring. Am J Med Genet. 1995; 59:536–545. [PubMed: 8585581]

Tamura T, Picciano MF. Folate and human reproduction. Am J Clin Nutr. 2006; 83:993–1016. [PubMed: 16685040]

van Driel LM, de JR, Helbing WA, van Zelst BD, Ottenkamp J, Steegers EA, Steegers-Theunissen RP. Maternal global methylation status and risk of congenital heart diseases. Obstet.Gynecol. 2008; 112:277–283. [PubMed: 18669723]

Wang L, Wang F, Guan J, Le J, Wu L, Zou J, Zhao H, Pei L, Zheng X, Zhang T. Relation between hypomethylation of long interspersed nucleotide elements and risk of neural tube defects. Am J Clin Nutr. 2010; 91:1359–1367. [PubMed: 20164316]

Waterland RA, Jirtle RL. Transposable elements: targets for early nutritional effects on epigenetic gene regulation. Mol.Cell Biol. 2003; 23:5293–5300. [PubMed: 12861015]

Weisenberger DJ, Campan M, Long TI, Kim M, Woods C, Fiala E, Ehrlich M, Laird PW. Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res. 2005; 33:6823–6836. [PubMed: 16326863]

Wilhelm CS, Kelsey KT, Butler R, Plaza S, Gagne L, Zens MS, Andrew AS, Morris S, Nelson HH, Schned AR, Karagas MR, Marsit CJ. Implications of LINE1 methylation for bladder cancer risk in women. Clin Cancer Res. 2010; 16:1682–1689. [PubMed: 20179218]

Yang AS, Estecio MR, Doshi K, Kondo Y, Tajara EH, Issa JP. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res. 2004; 32:e38. [PubMed: 14973332]

Yideng J, Jianzhong Z, Ying H, Juan S, Jinge Z, Shenglan W, Xiaoqun H, Shuren W. Homocysteine-mediated expression of SAHH, DNMTs, MBD2, and DNA hypomethylation potential pathogenic mechanism in VSMCs. DNA Cell Biol. 2007; 26:603–611. [PubMed: 17688412]

Yoon PW, Rasmussen SA, Lynberg MC, Moore CA, Anderka M, Carmichael SL, Costa P, Druschel C, Hobbs CA, Romitti PA, Langlois PH, Edmonds LD. The National Birth Defects Prevention Study. Public Health Rep. 2001; 116 Suppl 1:32–40. [PubMed: 11889273]

Zeschnigk M, Bohringer S, Price EA, Onadim Z, Masshofer L, Lohmann DR. A novel real-time PCR assay for quantitative analysis of methylated alleles (QAMA): analysis of the retinoblastoma locus. Nucleic Acids Res. 2004; 32:e125. [PubMed: 15353561]

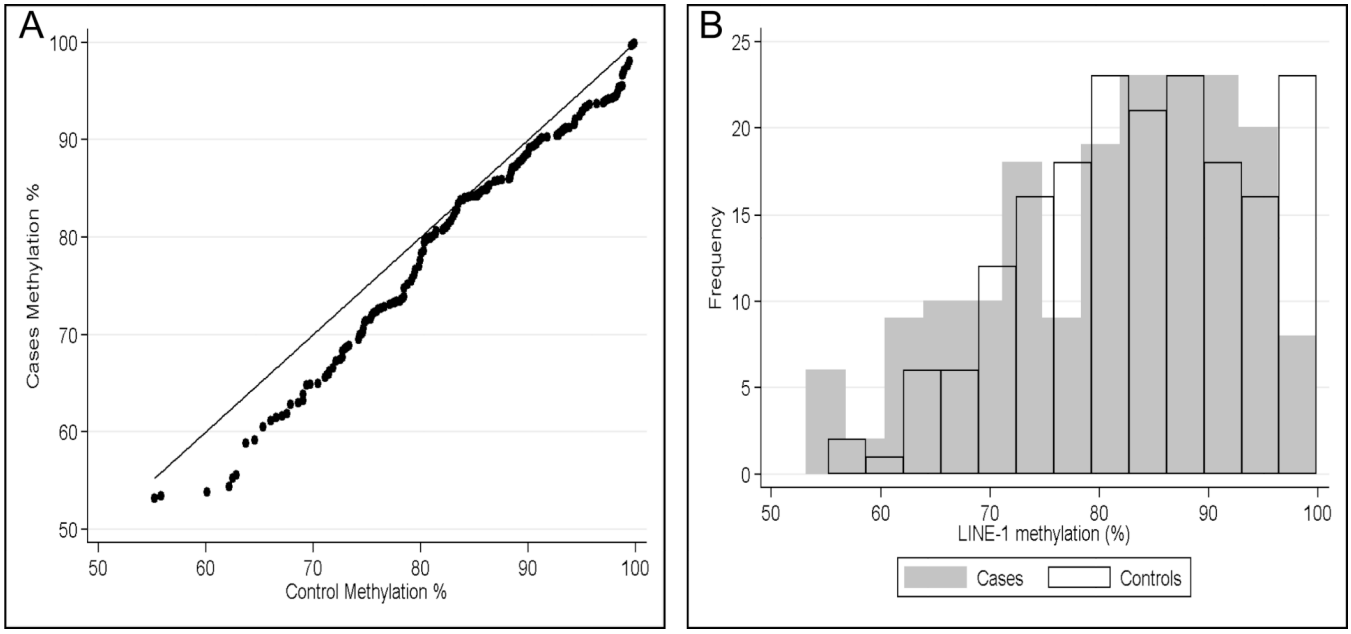

Figure 1. Quantile-quantile graph and frequency histogram of long interspersed nucleotide elements (LINE-1) methylation by case/control status

A) A quantile-quantile plot showing that the distribution of quantiles between cases and controls deviates from equality towards the lower values of LINE-1 methylation. Deviations from the line indicate different distributions among the two groups. B) A frequency histogram showing that cases and controls are distributed differently at higher and lower LINE-1 methylation values. Cases are more prevalent at lower methylation values, and controls are more prevalent at higher methylation values.

Table 1

Selected characteristics of cases and controls

	Cases (n=180) n (%)	Controls (n=187) n (%)	P value[a]
Age (y)			
<30	111 (61.7)	113 (60.4)	
≥30	69 (38.3)	74 (39.6)	0.831
Race			
Caucasian	136 (75.6)	148 (79.1)	
African American	32 (17.8)	22 (11.8)	
Others	12 (6.7)	17 (9.1)	0.205
Smoker			
Missing	0 (0.0)	1 (0.5)	
No	126 (70.0)	151 (80.7)	
Yes	54 (30.0)	35 (18.7)	0.015
Alcohol drinker			
Missing	1 (0.6)	2 (1.1)	
No	93 (51.7)	85 (45.4)	
Yes	86 (47.8)	100 (53.5)	0.294
Vitamin supplementation			
Missing	0 (0.0)	1 (0.5)	
No	99 (55.0)	111 (59.4)	
Yes	81 (45.0)	75 (40.1)	0.400
Maternal education			
Missing	5 (2.8)	0 (0.0)	
High school or less	83 (46.1)	79 (42.2)	
College education or higher	92 (51.1)	108 (57.8)	0.342
Household income			
Missing	13 (7.2)	12 (6.4)	
Less than $10,000	34 (18.9)	23 (12.3)	
$10,000–$30,000	60 (33.3)	55 (29.4)	
$30,001–$50,000	39 (21.7)	51 (27.3)	
More than $50,000	34 (18.9)	46 (24.6)	0.137
Body Mass Index (BMI) (kg/m^2)			
Missing	8 (4.4)	9 (4.8)	
Underweight (<18.5)	3 (1.7)	6 (3.2)	
Normal (18.5–24.9)	57 (31.7)	78 (41.7)	
Overweight (25.0–29.9)	44 (24.4)	34 (18.2)	
Obese (≥30.0)	68 (37.8)	60 (32.1)	0.118

[a] Fisher's exact test for categorical variables.

Table 2

Plasma biomarker concentrations for the comparison between cases and controls[a]

	Cases		Controls		
Biomarker	**N**	**Mean (SD)**	**N**	**Mean (SD)**	**P value[b]**
Homocysteine (μmol/L)	180	9.26 (2.78)	187	7.31 (1.67)	<0.0001
Methionine (μmol/L)	180	22.05 (5.27)	187	25.58 (4.69)	<0.0001
SAM (nmol/L)	180	74.48 (16.87)	187	82.13 (13.91)	<0.0001
SAH (nmol/L)	180	30.01 (12.00)	187	23.32 (7.96)	<0.0001
SAM/SAH	180	2.88 (1.38)	187	3.91 (1.38)	<0.0001
Folate (mg/L)	180	11.04 (4.08)	187	12.58 (5.69)	0.0311

[a] SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine

[b] Estimated by multiple linear regression model adjusted for age, race, vitamin supplementation, smoking, alcohol consumption, and body mass index (BMI); SD = standard deviation.

Table 3

LINE-1 methylation values and crude and adjusted P-values for the comparison between overall cases, specific cardiac phenotypes and controls

	N	Mean (SD)	Median (Min, Max)	P value[a]	P value[b]
Control	187	83.64 (10.16)	84.19 (55.24, 99.89)		
Cases	180	80.94 (11.28)	83.92 (53.21, 99.89)	0.049	0.010
Conotruncal	30	80.02 (11.20)	80.23 (53.21, 97.55)	0.110	0.085
Septal	85	81.26 (11.44)	84.02 (53.43, 99.89)	0.187	0.043
Obstructive	63	80.48 (11.76)	84.76 (53.85, 98.07)	0.125	0.086

[a] Two-sample Wilcoxon test comparing methylation value between case, each phenotype and controls.

[b] Estimated by multiple logistic regression model after adjusted for age, race, vitamin supplementation, smoking, alcohol consumption, and body mass index (BMI); SD=standard deviation.

Table 4

Adjusted OR and 95% CI for the association between case and control status at long interspersed nucleotide elements (LINE-1) methylation cutoffs

LINE-1 Methylation (decile)	Cases n (%)	Controls n (%)	Adjusted OR[a] (95% CI)
<78.5 (30th)	64 (35.6%)	58 (31.0%)	1.24 (0.78, 1.96)
<74.7 (20th)	55 (30.6%)	38 (20.3%)	1.72 (1.04, 2.84)
<69.9 (10th)	33 (18.3%)	20 (10.7%)	1.91 (1.03, 3.58)

[a] Adjusted for age, race, vitamin supplementation, smoking, alcohol consumption, and BMI. OR=Odds Ratio; 95% CI=95% Confidence Interval.

Table 5

Spearman partial correlation between long interspersed nucleotide elements (LINE-1) methylation and selected biomarkers[a]

	LINE-1 Methylation	
Biomarker	**Correlation Coefficient**	**P-value[b]**
Homocysteine	−0.135	0.013
Methionine	0.099	0.069
SAM	0.007	0.903
SAH	−0.067	0.216
SAM/SAH	0.048	0.381
Folate	0.057	0.294

[a] SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

[b] Adjusted for age, race, vitamin supplementation, smoking, alcohol consumption, and body mass index (BMI).